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High Performance Liquid Chromatographic Analysis of 1-Alkyl-2-acyl- and 1-Alkyl-3-acyl-*sn*-glycerols[1]

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A high performance liquid chromatographic (HPLC) method is described for separation and quantitation of 1-alkyl-3-acyl- and 1-alkyl-2-acyl-*sn*-glycerol, products of the detritylation reaction of 1-alkyl-2-acyl-3-trityl-*sn*-glycerol. The alkyl glycerides were separated on a 25 cm × 4.6 mm ID column packed with ~5–6 μm silica and eluted isocratically with isooctane/isopropanol (98:2, v/v) as mobile phase. The good separation and linear refractive index (RI) detector responses using cholesterol as an internal standard indicated the applicability of the method not only for the quantitative determination of the alkylglycerols but also for their semipreparative isolation. This HPLC method shows excellent reproducibility and accuracy and is applicable to other types of glycerides such as mono- and diacylglycerols.
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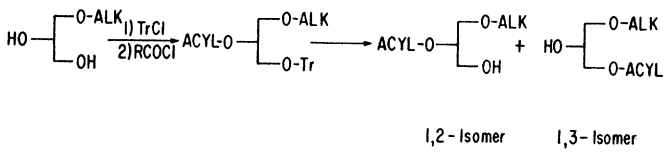
Common precursors for the stereospecific synthesis of 1-alkyl- neutral and phospholipids are 1-alkyl-2-acyl-3-trityl-*sn*-glycerols in which the trityl group is used to protect the hydroxyl group at the 3-position of glycerol (1,2). The next step in the synthetic sequence is removal of the trityl protecting group, under general acid catalysis, to yield 1-alkyl-2-acyl-*sn*-glycerols as key intermediates (Scheme 1). Acylation or phosphorylation of the latter glycerides gives the desired 1-alkyl- neutral or phosphoglyceride, respectively. Quite often, however, removal of the trityl protecting group is complicated by the concomitant migration of the 2-acyl moiety to yield the isomeric 1-alkyl-3-acyl-*sn*-glycerol derivative (3–6). As a consequence of this acyl rearrangement, it is necessary to separate and quantify these isomeric alkyl glycerides prior to further modification. A number of analytical methods, including column chromatography (7), thin layer chromatography (TLC) (8,9), high performance liquid chromatography (HPLC) (10,11) and gas liquid chromatography (GLC) (12) have been reported for separation and quantitative determination of mixtures of mono-, di- and triacylglycerols. For the isolation of glycerides for subsequent chemical manipulation, however, HPLC methods are more advantageous than other analytical methods because of their nondestructive nature, greater range of sample capacity and ease or rapidity of operation. Moreover, HPLC has the potential not only for isolating partial glycerides from reaction mixtures but also for their subsequent purification. This paper describes the separation and quantitative determination of mixtures of various 1-alkyl-2-acyl- and 1-alkyl-3-acyl-*sn*-glycerols as well as their semipreparative isolation by normal phase HPLC with isocratic elution.



SCHEME 1

MATERIALS AND METHODS

Materials. 1-Alkyl-2-acyl-3-trityl-*sn*-glycerols were synthesized in our laboratory starting with D-mannitol (99%) obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Oleic acid (>99.6%) used for the synthesis of oleoyl chloride (12) for acylation of the secondary hydroxyl group of glycerol was from Nippon Oil and Fats Co. (Amagasaki, Japan). Acetyl chloride (98.5%) was obtained from J.T. Baker Chemical Co. (Philipsburg, New Jersey); palmitoyl chloride (98%) and benzoyl chloride (99%) were obtained from Aldrich. Stearoyl chloride was synthesized in our laboratory. Triphenylmethanol used for the synthesis of triphenylchloromethane for tritylation of the primary hydroxyl group of glycerol was obtained from Eastman Organic Chemicals (Rochester, New York). The detritylation catalyst, boron trifluoride etherate, was obtained from Eastman Kodak Co. (Rochester, New York) and distilled prior to use. Isooctane and isopropanol used for HPLC separations were obtained from American Burdick & Jackson (Muskegon, Michigan). Cholesterol standard (>98%) was obtained from NuChek Prep (Elysian, Minnesota).

Analytical system. (a) Chromatograph: The solvent delivery system consisted of a Beckman Model 110A solvent delivery module equipped with a Waters Differential Refractometer Model R401 detector (Waters Associates, Milford, Massachusetts) and an Altex 210 injector. (b) Analytical HPLC column: Zorbax SIL, 4.6 mm ID × 25 cm (~5–6 μm, DuPont Co., Wilmington, Delaware). Sepralyte Diol, 4.6 mm ID × 25 cm (5 μm, Analytichem International, Harbor City, California). Semipreparative HPLC column: Dynamax Prepacked Silica Column, 10 mm ID × 25 cm (8 μm, Rainin Instrument Co., Woburn, Massachusetts). (c) Integrator and recorder: Chromatopac C-R3A (Shimadzu Co., Columbia, Maryland). (d) HPLC conditions: The samples were eluted isocratically with 98% isooctane/2% isopropanol (v/v) at a flow rate of 1 ml/min (analytical HPLC) or 3 ml/min (semipreparative HPLC). Injection volumes were 20 μl (analytical HPLC) or 100 μl (preparative HPLC), and the samples were injected via loop injectors.

Syntheses. 1,2-Isopropylidene-*sn*-glycerol was prepared from D-mannitol via the lead tetraacetate cleavage of 1,2,5,6-diisopropylidene-D-mannitol to 1,2-isopropylidene-*sn*-glyceraldehdyde and subsequent reduction of the aldehyde with sodium borohydride according to the procedure established by Eibl (14). $[\alpha]_D^{25}$ + 14.5° (0.169 mg/ml, CH_3OH). Infrared spectrum: 3425 cm^{-1} (OH), 1378 and 1368 cm^{-1} (isopropyl).

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3-Hexadecyl-*sn*-glycerol was prepared from 1,2-isopropylidene-*sn*-glycerol by reaction with sodium hydride in dimethylformamide followed by alkylation with hexadecylbromide, mp 64.9–67.0 C, lit. mp 65–67 C (13). Infrared spectrum 3405, 3326 and 3240 cm^{-1} (OH), 1127 cm^{-1} (C-O-C).

3-Hexadecyl-*sn*-glycerol was converted to its enantiomer, 1-hexadecyl-*sn*-glycerol, according to the method of Chacko and Hanahan (15). Reaction of 3-hexadecyl-*sn*-glycerol with p-toluene-sulfonyl chloride yielded the ditosylate derivative, which underwent SN_2 displacement with acetate anion to give 1-hexadecyl-2,3-diacetoxy-*sn*-glycerol. Alkaline hydrolysis of the diacetate gave the desired 1-hexadecyl-*sn*-glycerol, mp 64.4–66.0 C, lit. mp 65–67 C (15). Infrared spectrum: 3405, 3333 and 3240 cm^{-1} (OH), 1130 cm^{-1} (C-O-C).

1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol was synthesized by tritylation at the 3-position of 1-hexadecyl-*sn*-glycerol followed by acylation at the 2-position of the resultant 1-hexadecyl-3-trityl-*sn*-glycerol (16). Infrared spectrum: 3085, 3055 and 3020 cm^{-1} (triphenylmethyl), 3000 cm^{-1} (-CH=CH-), 1733 cm^{-1} (-C=O). Other 1-hexadecyl-2-acyl-3-trityl-*sn*-glycerols were prepared in a similar manner.

Detritylation reaction. The detritylation reaction was carried out using a modification of Hermetter and Pattauf's method (16). 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol (40 μmol), BF_3-CH_3OH catalyst (40 μmol) and methylene chloride (2 ml) were placed into a 4-ml glass vial equipped with a magnetic stirrer. After the vial was sealed with a viton seal under nitrogen, the mixture was stirred for ~30–60 min at 22 C. Similar reactions were performed with other 1-hexadecyl-2-acyl-3-trityl-*sn*-glycerols having various acyl groups at the 2-position (Table 2). The corresponding detritylation products, 1-hexadecyl-2-acyl-*sn*-glycerol and 1-hexadecyl-3-acyl-*sn*-glycerol were isolated by semipreparative HPLC described above.

Internal standard calibration. Standards containing 10 to 40 μmol of 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 1-hexadecyl-2-oleoyl-*sn*-glycerol, 1-hexadecyl-3-oleoyl-*sn*-glycerol and cholesterol (internal standard) were prepared in 1 ml of isooctane/isopropanol (98:2, v/v). The diradyl glycerol solution and standard cholesterol solutions were mixed (~50–200 μl) and analyzed by analytical HPLC. The weight ratios (w/w) of the alkyl glycerol (W) to the internal standard (WS) were varied between 0.1 and 1.4. Similar analyses were performed for the other diradyl glycerols having different acyl groups at the 2-position (Table 2).

RESULTS AND DISCUSSION

1-Alkyl-2-acyl glycerols are one of the more important classes of intermediates for the syntheses of 1-alkyl phospholipids. Much effort has been given to the synthesis and characterization of this class of lipid since migration of the 2-acyl unit to more thermodynamically stable 1,3-isomer is very facile. It is well known that the isomerization of the 1,2-isomer to 1,3-isomer is subject to general acid or base catalysis (17). As a result of this rearrangement, rapid methods are needed for analysis and purification of these partial glycerides. In this study, we have established an excellent separation method for this class of alkyl glycerol by normal phase HPLC.

HPLC and internal standard. HPLC separations of a test mixture consisting of (a) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, (b) triphenylmethyl alcohol, (c) 1-hexadecyl-3-oleoyl-*sn*-glycerol, (d) 1-hexadecyl-2-oleoyl-*sn*-glycerol and (e) cholesterol (IS) are shown in Figure 1. As the chromatograms indicate, some differences can be seen in the separations between the silica column and the diol column used in this study. The difference in retention times between the 1,3- and 1,2-isomers found in the HPLC on silica was larger than on the diol column, and no peaks for detritylation reaction products were found after elution of the 1,2-isomer. The data suggested the possibility of using a silica HPLC separation for the quantitative analysis of detritylation reaction mixtures and for preparative HPLC. Cholesterol is a suitable internal standard for quantitation.

Figure 2 shows the effect of solvent strength on the separation of a test mixture. As the results indicate, a better resolution of the five components in the test mixture was obtained with an increase in the amount of isooctane in the solvent system. However, it should be noted that the decreased solubility of triphenylmethanol with this mobile phase composition caused deterioration of the HPLC column and interfered with the HPLC analysis. The efficiency of the column was restored after washing it with 90:10 isooctane/isopropanol. Accordingly, it seems that a solvent system of isooctane/isopropanol of 98:2 composition is the preferred solvent system.

Figure 3 gives the RI detector responses of (a) 1-hexadecyl-2-oleoyl-*sn*-glycerol, (b) 1-hexadecyl-3-oleoyl-*sn*-glycerol and (c) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol. The

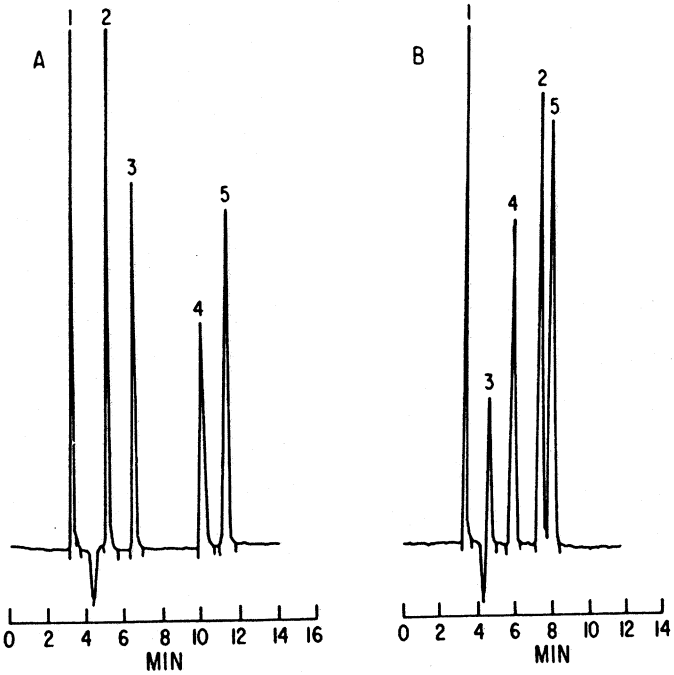

FIG. 1. HPLC chromatograms of a test mixture. Panel A, silica column; panel B, diol column. Compounds separated: 1) 1-hexadecyl-2-oleyl-3-trityl-*sn*-glycerol, 2) triphenylmethanol, 3) 1-hexadecyl-3-oleoyl-*sn*-glycerol, 4) 1-hexadecyl-2-oleoyl-*sn*-glycerol and 5) cholesterol.

data are plotted as weight ratio of the alkylglycerol to the internal standard (W/W_{IS}) along the ordinate and HPLC area ratios (A/A_{IS}) along the abscissa. In the case of the RI detector, good linear relation and sensitivity were obtained in the weight ratio range of 0 to 1.2 (alkylglycerol/standard). Detector response factors were calculated from the slope of each line, and the standard deviations obtained for the four different test mixtures (Table 1) (A, 1.7%; B, 1.3%; C, 1.4%, respectively) indicate that the method has good reproducibility and accuracy for the quantitative analysis of alkylglycerols.

In the quantitative analysis of the detritylation reaction, the mole fraction of each material was calculated from the following equation, where each weight ratio was obtained from each HPLC area ratio using the response factors given in Figure 3.

$$F_A = \frac{\frac{WA/WIS}{MA}}{\frac{WA/WIS}{MA} + \frac{WB/WIS}{MB} + \frac{WC/WIS}{MC}}$$

where F is the mole fraction of the alkylglycerol in the reaction mixture. WA/WIS, WB/WIS and WC/WIS are the weight ratios of materials A, B and C, respectively. MA, MB and MC are the molecular weights of materials A, B and C, respectively.

Retention time of alkylglycerols. The retention times obtained using the conditions given in Materials and Methods for 1-hexadecyl-2-acyl-3-trityl-, 1-hexadecyl-3- and 1-hexadecyl-2-acyl-*sn*-glycerols containing various fatty acyl chains are given in Table 2. As the data show, the retention times of the alkylglycerols increased with a decrease of the carbon chain length of the fatty acyl residue due to an increase of its polarity. Separation of mixtures of various molecular species for each lipid class

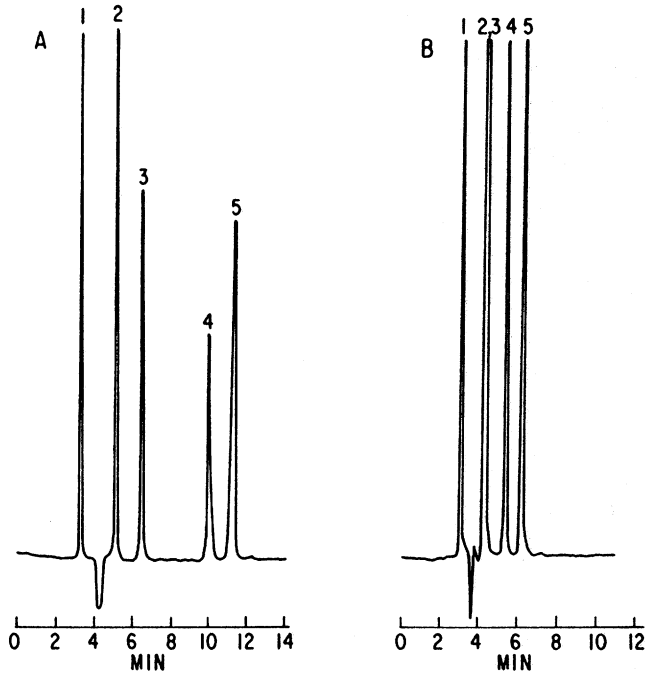

FIG. 2. Effect of solvent on the separation of a test mixture. A) Isooctane/isopropanol, 98:2 (v/v); B) isooctane/isopropanol, 95:5 (v/v). 1) 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 2) triphenylmethanol, 3) 1-hexadecyl-3-oleoyl-*sn*-glycerol, (4) 1-hexadecyl-2-oleoyl-*sn*-glycerol and 5) cholesterol.

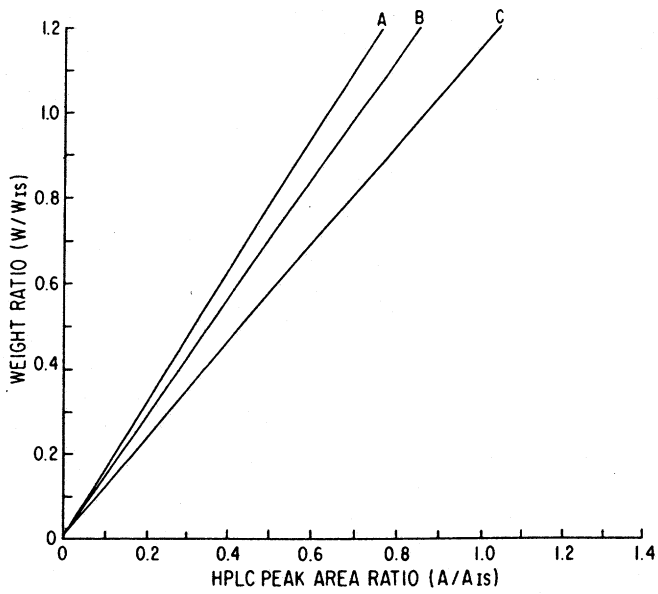

FIG. 3. Internal standard calibrations for A) 1-hexadecyl-2-oleoyl-*sn*-glycerol, B) 1-hexadecyl-3-oleoyl-*sn*-glycerol and C) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol.

TABLE 1

Quantitative Analyses of Alkylacylglycerols

Standard mixture	A: 1,2-(MF)[a]			B: 1,3-(MF)			C: 1,2,3-(MF)		
	Found[b]	Actual[c]	Ratio	Found	Actual	Ratio	Found	Actual	Ratio
Sample A	0.394	0.387	1.018	0.378	0.381	0.992	0.229	0.232	0.987
Sample B	0.445	0.438	1.016	0.428	0.430	0.995	0.126	0.131	0.962
Sample C	0.483	0.479	1.008	0.232	0.235	0.987	0.286	0.286	1.000
Sample D	0.234	0.240	0.975	0.482	0.472	1.021	0.285	0.288	0.990
Mean ± S.D. ± 0.014		1.004 ± 0.017			0.999 ± 0.013			0.985	

[a]MF, mole fraction. Average of three observations. A, 1-Hexadecyl-2-oleoyl-*sn*-glycerol; B, 1-hexadecyl-3-oleoyl-*sn*-glycerol; C, 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol.

[b]Mole fraction determined by semipreparative HPLC isolation.

[c]Mole fraction determined by analytical HPLC using response factors given in Fig. 3.

TABLE 2

HPLC Retention Times of Glycerides Containing Various Fatty Acids

Fatty acid at 2-position	Retention time (min)[a]		
	1,2,3[b]	1,3[c]	1,2[d]
Acetic	3.94	18.04	27.84
Benzoic	3.58	9.38	14.22
Palmitic	3.19	6.85	10.92
Stearic	3.16	6.50	10.16
Oleic	3.17	6.56	10.21
Oleic[e]	3.37	9.08	13.28

[a]Average of three observations—analytical HPLC silica column: 98:2 (v/v) isooctane/isopropanol, flow rate of 1 ml/min.

[b]1-Hexadecyl-2-acyl-3-trityl-*sn*-glycerol.

[c]1-Hexadecyl-3-acyl-*sn*-glycerol.

[d]1-Hexadecyl-2-acyl-*sn*-glycerol.

[e]1,2,3-, triolein; 1,3- and 1,2-, diolein.

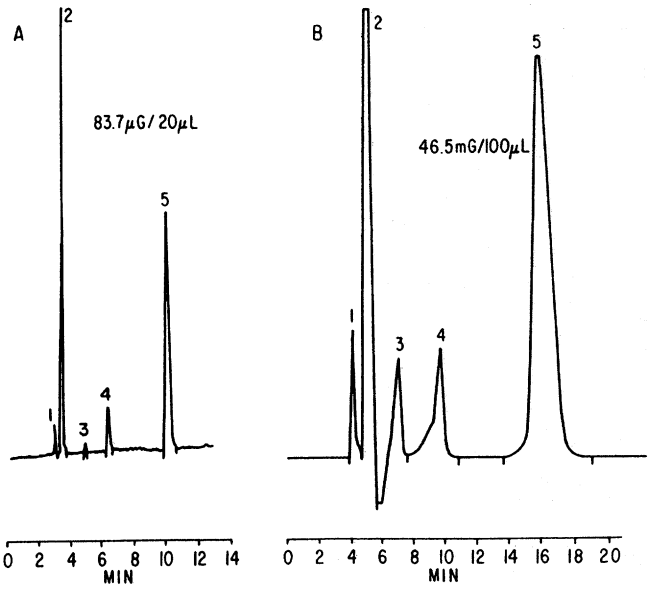

FIG. 4. HPLC chromatograms of detritylation reaction product. A) Analytical HPLC silica column—98.2 (v/v) isooctane/isopropanol, flow of 1.0 ml/min; B) semipreparative HPLC silica column—98:2 (v/v) isooctane/isopropanol, flow of 3 ml/min. 1) 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 2) methyl triphenylmethyl ether, 3) triphenylmenthanol, 4) 1-hexadecyl-3-oleoyl-*sn*-glycerol and 5) 1-hexadecyl-2-oleoyl-*sn*-glycerol.

was not always possible with the HPLC conditions used. However, a complete separation of individual isomeric lipid species, 1,2,3-, 1,3- and 1,2-glycerols, was obtained under the conditions. The separation among these three derivatives increased with a decrease in the carbon chain of fatty acid acyl residue, with the largest separations obtained with the acetyl derivatives. The last entry in Table 2 shows that the method is also applicable to the separation of mono-, di- and triacylglycerols, giving the same order of elution as observed with the alkylacylglycerols. However, substitution of an acyl residue for alkyl increases retention times for all three acyl glycerides. This indicates the decreased polarity of an alkoxy group compared to an acyloxy substituent.

Preparative HPLC application. To obtain information on the utility of preparative column HPLC for the isolation of the 1,2- and 1,3-alkylacyl isomers, both the analytical and preparative HPLC columns were compared at different sample loadings (Fig. 4). The sample used for these chromatograms was obtained by the detritylation reaction of 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol at a reaction temperature of 22 C, in methylene chloride solvent, BF_3-CH_3OH to glyceride molar ratio of 1:1 and a reaction time of 5 min. As the data show, a comparable separation and sensitivity was obtained on the semipreparative silica column as with the analytical silica column. This result suggested the further possibility of using HPLC for the semipreparative isolation and purification of the isomeric 1-alkyl-2-acyl and 1-alkyl-3-acyl-*sn*-glycerols listed in Table 2.

At the present stage of the study, only the semipreparative column was used for this purpose. In the future, we are planning to use preparative columns with larger ID for isolation of larger amounts of synthetic mixed alkylacylglycerol isomers.

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